Genetic and proteomic analysis of *Candida albicans* biofilm

Synopsis
Submitted to

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For the registration of Doctor of Philosophy
In the Biotechnology

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Introduction:

Candidiasis or candidosis are the infections caused by *Candida* sp. The *Candida* genus is a member of the natural microbiota of the human mucosal, oral cavity, gastrointestinal tract and vagina (Sardi et al., 2013). However, some of the commensal members of genus *Candida*, can become opportunistic pathogens when induced by changes in the host microenvironment (Papon et al., 2013). Immunocompromised patients are more susceptible to develop both superficial and life-threatening *Candida* infections (Sardi et al., 2013). Currently candidiasis represents the fourth main reason of nosocomial infections (10%) with a high mortality (15-35%) depending on the infections of *Candida* species (Sadeghi et al., 2018). There are fifteen *Candida* species known to be pathogenic for humans. However, more than 90% reported invasive candidiasis are associated with the five most common species i.e. *C. albicans, C. glabrata, C. parapsilosis, C. krusei* and *C. tropicalis* (Pappas et al., 2016). The incidence of disseminated candidiasis according to population-based studies differ within various geographic areas (Sampaio & Pais 2014). In the USA, Europe and Latin America these values ranged from 0.28-0.96, 0.1-0.53 and 0.33-1.96 episodes/1,000 admissions respectively (Sampaio & Pais 2014). There are three major types of Candidiasis: cutaneous (skin and its appendages), mucosal (oro-pharyngeal, esophageal, and vulvovaginal) and systemic (bloodstream infections) (Sadeghi et al., 2018).

Four major classes of antifungal drugs used for treatment of fungal infections viz; azoles (such as fluconazole), polyenes (such as amphotericin B), echinocandins (such as caspofungin) and nucleoside analogues (such as 5-flucytosine (5-FC)) (Fox et al., 2015). The azoles inhibits the ergosterol synthesis, whereas polyenes bind to sterols and lead to intracellular leakage (Chong et al., 2018). Echinocandins inhibit β-1,3-glucan synthase which disrupt cell wall integrity.

Nucleoside analogues specifically acts as a pyrimidine analogue, and disrupts fungal RNA, DNA, and protein biosynthesis (Prasad et al., 2016).

C. albicans is a common opportunistic fungal pathogen of humans and it is a diploid yeast with two pairs of 8 chromosomes, possesses 6,159 coding genes and its genome size is about 16 Mb (Dabas et al., 2013; Lu et al., 2014). *C. albicans* asymptomatically colonize reproductive tract, oral cavity, gastrointestinal (GI) tract and skin of most humans (Nobile & Johnson 2015). However, *C. albicans* become infectious pathogen when changes in host immunity, physiology, and/or micro-biota happened (Lu et al., 2014). Hundreds of millions of symptomatic infections each year is caused by *C. albicans* and it also main cause of nosocomial fungemia (Witchley et al., 2019; Tong & Tang 2017). Almost 80% of total fungal bloodstream infections are associated with *C. albicans* (Thakre et al., 2018). Superficial mucocutaneous infections of genital (vulvovaginal, balanitis and balanoposthitis and candiduria) and non-genital (Oropharyngeal) tracts of healthy individuals is one of the most important infections caused by *C. albicans* (Thakre et al., 2016). Life-threatening illness can cause by *C. albicans* with significant rates of mortality in immunocompromised patients and in patients receiving immunosuppressive therapy (Tong & Tang 2017; Zore et al., 2011).

The pathogenicity of *C. albicans* supported by a wide range of virulence factors and fitness attributes (Nicholls et al., 2011). There are several virulence factors such as polymorphism, phenotypic switching, adhesins, invasins, biofilm, thigmotropism and hydrolytic enzymes (Dadar et al., 2018; Mayer et al., 2013; Sardi et al., 2013; Mallick et al., 2016). Biofilm formation is considered as one of the major virulence factors contributing to the pathogenicity of *C. albicans* (Fernandes et al., 2015). The formation of biofilms enable *C. albicans* to cause clinical problems of the host (Sardi et al., 2013). Biofilm formation is regulated by several transcription factors, including those involved in adhesion, hyphal formation, and extracellular

polymeric substances (EPS) production (Cavalheiro & Teixeira 2018). More than 50 transcriptional regulators have been linked to *C. albicans* biofilm formation (Lohse et al., 2018). This transcriptional regulators is comprised of six "master" transcriptional regulators (Efg1, Tec1, Bcr1, Ndt80, Brg1, and Rob1) that is required for biofilm development (Gulati & Nobile 2016). Biofilm provide *C. albicans* significant resistance to antifungal agents, evading host immune mechanisms and protection against other chemical and physical stresses (Gonçalves et al., 2020). In present study, we are planning to understand the regulation of biofilm formation using genomic and proteomic approach.

Literature review

Formation of *C. albicans* biofilm:

Biofilm is a surface-associated microbial communities typically surrounded in a matrix of extracellular polymeric substances (Wall et al., 2019). Overall, the *C. albicans* biofilm form in a sequential process can be divided into four major phases including: adherence, proliferation, maturation, and dispersal (Tsui et al., 2016). In the first step of infection, adhesion, the yeast cells bind to the ligand on the surface of host cells through cell wall glycoproteins called adhesins (Nobile et al., 2012). Proliferation phase (Initiation step) produce elongated projections that grow into filamentous forms, leading to the emergence of hyphal and pseudohyphal cells (Finkel & Mitchell 2011). In the maturation phase an accumulation of extracellular matrix material occur as the biofilm matures, and high-level drug resistance is also acquired (Mayer et al., 2013). This extracellular matrix composed of glycoproteins (55%), carbohydrates (25%; largely α-mannan and β-1,6-glucan polysaccharides, and, to less extent, β-1,3-glucan), lipids (15%) and nucleic acids (5%) (Pierce et al., 2017). Finally, dispersion of

yeast cells from the biofilm complex into the surrounding environment and initiate the formation of new biofilms (Tournu & Van 2011).

Clinical significance of biofilm

The formation of biofilm increase the pathogenicity of *C. albicans* and also re-infection may occur because biofilm may act as reservoirs of infectious cells (Gonçalves et al., 2020; Somwanshi Sachin et al., 2019; Fernandes et al., 2015). Many different mechanisms exhibited by *C. albicans* biofilms contributes to much more resistance to antifungal drug (Wall et al., 2019). Briefly, the extracellular matrix, overexpression of drug efflux pumps, metabolic plasticity, existence of persister cells and alter in the sterol composition of the cell membrane (Mayer et al., 2013; Wall et al., 2019). The extracellular matrix in biofilm act as physical barrier and sequester antifungal drug (Nobile & Johnson 2015). Two main classes of efflux pumps in *C. albicans*, are the major facilitator (MF) transporter superfamily (including Mdr1) and the ATP binding cassette (ABC) transporter superfamily (including Cdr1 and Cdr2) (Fox et al., 2015). In planktonic cells several efflux pumps are upregulated in response to antifungal drugs (Lohse et al., 2018). While, in biofilms, efflux pumps are upregulated earlier in adhesion and remain upregulated during biofilm development process, even in the absence of an antifungal drug (Gulati & Nobile 2016). The existence of persister cells (minority cell population) consider as another factor which plays role in drug resistant in *C. albicans* biofilm (Mathé & Van 2013). The persister cells are non-dividing cells with decreased metabolic activities increase the resistance of biofilms to antimicrobial drugs and further, likely to seed new biofilm infections after drug treatment (Lohse et al., 2018).

- ## Regulation of biofilm development

The major difference between planktonic and biofilm cells is that the formation of *C. albicans* biofilm is regulated by more than 50 transcriptional regulators (Lohse et al., 2018). These

transcriptional regulators which controls the development of *C. albicans* biofilm consists of six master transcriptional regulators (Efg1, Tec1, Ndt80, Bcr1, Brg1, and Rob1) (Chong et al., 2018). Furthermore, these six transcription factors were found to regulate a high degree of overlapping target genes as well as to control the expression of each other (Cavalheiro & Teixeira 2018). Thus, these six master regulators are regulating the expression of approximately 1,000 target genes (Fox et al., 2015). In addition, 44 transcriptional regulators involved in regulation of biofilm formation have been identified (Gulati & Nobile 2016). The majority of these 44 transcriptional regulators are directly bind to at least one of the 6 master biofilm network regulators, indicating that they are having direct regulatory role in the biofilm formation (Nobile & Johnson 2015). A few years later, three new transcriptional regulators involved in the biofilm circuit include Rfx2, Gal4 and Flo8 have been identified which have specific roles in the development of the biofilm over time (Chong et al., 2018).

Objectives:

1. Optimization of *in vitro* biofilm formation.
2. Proteomic analysis of biofilm by Liquid chromatography and mass spectrometry (LC-MS).
3. Identification of biofilm specific proteins.
4. Expression analysis of selected genes during biofilm formation.

Significance of the study:

The biofilms cause clinical problems of concern because of significant resistance to antifungal agents and evading host immune mechanisms**.** The aim of this study is to understand the regulation of biofilm formation in order to identify novel drug targets for antifungal chemotherapy**.**

Materials and Method:

- ***Candida albicans* strain**

The standard strain of *C. albicans* ATCC 10231 is procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTech), Chandigarh, India. The strain is cultured on YEPD (1% yeast extract, 2% peptone, 2% dextrose and 2.5% agar) agar slants with pH 6.5 and maintained at 4 °C.

- **Preparation of inoculum**

Single colony from the yeast extract-peptone-dextrose (YEPD) agar plates is inoculated in 50 ml of YPD broth (pH 6.5), in a 250 mL erlenmeyer flask. The flasks incubate at 30° C on an orbital shaker at 120 rpm for 24 h. Then, cells will be harvested by centrifugation at 1000g for 2 min at room temperature; washed with sterile distilled water, and resuspended in phosphate buffered saline (PBS). Cell density is determined microscopically using hemocytometer and adjusted to 1×10^7 cells/ml. (Ahmed et al., 2018; Thakre et al., 2018)

- **Biofilm formation:**

Biofilms were induced as per the protocols of Li et al., 2003; Jin et al., 2004; Thakre et al., 2018.

- **Determination of biofilm production**
    1. A semi- quantitative measure of biofilm formation and viability (Hasan et al., 2009; Thakre et al., 2018; Gonçalves et al., 2020).
    2. Biofilm total biomass (Li et al., 2003; Fernandes et al., 2015; Gonçalves et al., 2020).
    3. Microscopic analysis: under an inverted light microscope.

- **Proteomic analysis of biofilm by (LC-MS) and identification of biofilm specific proteins.**

1. **Sample preparation for LC-MS/MS analysis** (Ahmed et al., 2018; Thakre et al., 2018; Seneviratne et al., 2008)

   - Extraction of proteins from biofilm.
   - Protein estimation: proteins will be estimated by Bradford method (Kruger 2009).
   - Trypsin digestion

2. **LC-MS/MS analysis of proteins**

Protein identification and relative quantification by SWATH-MS (Sequential window acquisition of all theoretical fragment ion spectra, strategy for high throughput labels free protein quantification) analysis by using mass spectrometer coupled with LC in high-sensitivity mode. For generation of spectral library equal amounts of digested peptides from each treatment were pooled together and analyzed via LC MS/MS in an Information dependent acquisition (IDA) mode.

- **Identification of biofilm specific proteins.**

  Comparison between proteins which identified in previous step with proteins existing in planktonic cells.

- **Expression analysis of selected genes during biofilm formation.**

  1. RNA extraction.
  2. cDNA synthesis.
  3. Real-time qPCR analysis of selected genes.

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